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Characterization of Chemically Tritiated Microcystin-LR and its Distribution in Mouse

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Chemically tritiated microcystin-LR (specific activity 194 mCi/mmol), purified to >95% by C-18 reverse-phase HPLC, exhibited the same retention time and ultraviolet absorption profile as unlabeled toxin. Acid-hydrolyzed [^{3}H]toxin yielded tritiated glutamate and β-methylasparate. Stability of the nonexchangeable [^{3}H]toxin in saline and urine was >93% after 42 days stored at 22°, 4°, or -20°C. In blood the breakdown of toxin was temperature- and time-dependent (63% at 22°C, 28 days). Unlabeled toxin was stable for 542 days stored at either 4° or -20°C in saline. The LD_{50} (mouse, i.p.) of [^{3}H]microcystin-LR & unlabeled toxin was the same (75 μg/kg [65-90] & 65 μg/kg [53-80], respectively). From 3-90 min after i.p. injection of 70 μg/kg [^{3}H]microcystin-LR there was a slow absorption of toxin from the peritoneal cavity & efficient accumulation in liver. The elimination half-life of the plasma concentration curve was 29 min. Tritium distribution in tissue at death or 6 hr post injection was similar for all doses (13-101 μg/kg). At 101 μg/kg, liver contained 56 ± 1%, intestine 7 ± 1%, kidney 0.9 ± 0.2% & carcass 10 ± 1% of the injected dose. Heart, spleen, lung and skeletal muscle contained <1% of the radiolabel.

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CHARACTERIZATION OF CHEMICALLY TRITIATED MICROCYSTIN-LR AND ITS DISTRIBUTION IN MOUSE

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The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense (para. 4-3, AR 360-5). In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

N. A. ROBINSON, G. Y. MIURA, C. F. MATSON, R. E. DINTERMAN, and J. G. PACE. Characterization of chemically tritiated microcystin-LR and its distribution in mouse. *Toxicon* . . 19 . Chemically tritiated microcystin-LR (specific activity 104 mCi/mmol), purified to >95% by C-18 reverse-phase high performance liquid chromatography, exhibited the same retention time and ultraviolet absorption profile as unlabeled toxin. Acid-hydrolyzed [^{3}H]toxin yielded tritiated glutamate and B-methylasparate. Stability of the nonexchangeable [^{3}H]toxin in saline and urine was >93% after 42 days stored at 22°, 4°, or -20°C. In blood the breakdown of toxin was temperature- and time-dependent (63% at 22°C, 28 days). Unlabeled toxin was stable for >42 days stored at either 4° or -20°C in saline. The LD_{50} (mouse, i.p.) of [^{3}H]microcystin-LR and unlabeled toxin was the same (75 µg/kg [65-90] and 65 µg/kg [53-80], respectively). From 3-90 min after i.p. injection of 70 µg/kg [^{3}H]microcystin-LR there was a slow absorption of toxin from the peritoneal cavity and efficient accumulation in liver. The elimination half-life of the plasma concentration curve was 29 min. Tritium distribution in tissue at death or 6 hr post injection was similar for all doses (13-101 µg/kg). At 101 µg/kg, liver contained 56 ± 1%, intestine 7 ± 1%, kidney 0.9 ± 0.2% and carcass 10 ± 1% of the injected dose. Heart, spleen, lung and skeletal muscle contained <1% of the radiolabel.

INTRODUCTION

Microcystin-LR (MCYST-LR), a member of a family of blue-green algal toxins (BOTES *et al.*, 1985), is a cyclic heptapeptide hepatotoxin (for review see CARMICHAEL, 1986). Little is known about the disposition and fate of these environmentally important toxins. These types of studies require radiolabeled toxin of high specific activity.

Two methods for radiolabeling MCYST toxins have been reported: [^{125}I]MCYST-YM (RUNNEGAR *et al.*, 1986, FALCONER *et al.*, 1986) and [^{14}C]MCYST (BROOKS and CODD, 1987). FALCONER *et al.* (1986) measured the distribution and biological half-lives (α phase 2.1 min, β phase 42 min) and the distribution of radiolabel (20% in liver, 6% in kidney, 9% gut contents, 3% in urine) in rat tissues after an i.v. injection of the iodinated toxin. Recovery of radioactivity was low (38% of dose) and deiodination of the radiolabeled compound was not addressed. The disadvantages of the iodinated compound are that the half-life of ^{125}I is short (60 days) and the iodination procedure is specific for the YM variant. BROOKS and CODD (1987) measured the disposition of biosynthetically labeled [^{14}C]MCYST in mice at 1-120 min post i.p. injection of a sub-LD_{50}. They reported an extraordinarily rapid accumulation of radiolabel in liver, 70% by 1 min, but did not measure plasma concentrations. Also the question of toxin metabolism and excretion was not addressed, probably because the biosynthetically obtained [^{14}C]toxin had a low specific activity, 2.6 μCi/mg.

Our goal is to define a kinetic model describing the disposition and metabolic fate of MCYST-LR in mouse. The information derived from these experiments should aid in design of therapeutic treatment for MCYST intoxication and diagnosis of toxicosis. Reported here are the purification and characterization of chemically radiolabeled [^{3}H]MCYST-LR, the stability of the compound in biological fluids, and the plasma concentration and distribution of radioactivity in mouse tissue after i.p. injection.

MATERIALS AND METHODS

Unlabeled MCYST-LR (>95% purity), isolated from *Microcystis aeruginosa* strain 7820, was supplied by Dr. Wayne W. Carmichael (Wright State University, Dayton, OH). [^{3}H]MCYST-LR (40% radiochemically pure), was prepared by Amersham Corp. (Arlington Heights, IL), by an exchange procedure (MATSUO and NARITA, 1975) by using ^3H_2O in the presence of acetic anhydride and pyridine.

Male VAF/plus CD-1 mice (Crl:CD-1(ICR)BR, Charles River, Wilmington MA) weighing 19-25 g were maintained on a 12 hr light/dark cycle. Mice were fasted the night before studies but allowed water *ad libitum*.

HPLC purification of [^{3}H]MCYST-LR

The radiolabeled toxin was purified with a Waters high performance liquid chromatography (HPLC) system (Milford, MA) equipped with a Waters 490 multiwavelength detector, essentially by the method of KRISHNAMURTHY *et al.*, (1986). A C-18 column (Adsorbosphere HS, 4.6 x 250 mm, 5 µm, Alltech, Deerfield, IL), had a flow rate of 1 ml/min of 75% 10 mM ammonium acetate, pH 6.0, and 25% acetonitrile for 20 min. Elution of toxin was monitored at 240 nm. The ratio of solvents varied from 77/23 to 73/27 and was selected to give a retention time for MCYST-LR of 8-12 min. Toxin was collected manually after injection of 60 ug of [^{3}H]MCYST-LR. After each injection, the column was washed for 10 min with a gradient from 25-100% acetonitrile. The gradient was held for another 10 min at 100% acetonitrile, then returned to starting conditions with a 10 min reverse gradient. Purified toxin was pooled and dried under a stream of N_2. Water was added to dried toxin, and the solution (1 mg/ml) was stored at -10°C. The specific activity of the purified material, measured by liquid scintillation and HPLC was 0.20 mCi/mg or 194

mCi/mmol. Radiochemical purity, 95%, was measured with a Flow 1 β-radiodetector (Radiomatic Instruments and Chemical Co., Tampa, FL), outfitted with a 5 ml cell with Flo-Scint III scintillation fluid (Radiomatic) pumped at 2 ml/min. The efficiency was 40%.

Characterization of [^{3}H]MCYST-LR

(a) Acid exchange: purified radiolabeled toxin in 10% glacial acetic acid (pH 2.35) was repetitively dried under a stream of N_2 at room temperature. Residual radioactivity was measured in a Beckman model 5800 liquid scintillation counter (Berkley, CA). (b) ultraviolet (UV) profile - tritiated (20 µg/ml) and unlabeled (25 µg/ml) toxin in H_2O were scanned from 195-335 nm with a DU-8 spectrophotometer (Beckman, Berkley, CA).

HPLC determination of [^{3}H]amino acids from acid hydrolyzed [^{3}H]MCYST-LR

[^{3}H]MCYST-LR (10 µg) in 6N HCl was heated for 24 hr at 110°C to hydrolyze the toxin to constituent amino acids. The mixture was dried in a rotary evaporator and 0.2 M sodium citrate, pH 3.28, was added to the dried sample. HPLC analysis was performed on a Beckman (Berkley, CA) system with System Gold software. The sample was injected onto a Sperogel AA-Na^+ column (3.0x250 mm) at a flow rate of 0.6 ml/min. The solvent system was 0.2 M sodium citrate, pH 3.28, for 19 min followed by 1.0 M sodium citrate, pH 7.4. Radioactivity was detected with a Beckman 171 Radioisotope Detector outfitted with a 200 µl liquid flow cell.

Stability of [^{3}H]MCYST-LR

Saline, and human urine and blood from a healthy 45 year old male, were spiked with purified [^{3}H]MCYST-LR (3.5×10^6 DPM/ml). Blood was collected in an evacuated tube containing NaF by venipuncture, and NaF (2.5%) was added to urine. Each sample was divided into three portions. One aliquot was stored at room temperature, one at 4°C, and the last was divided into 0.1 ml portions and stored at -20°C. After 1, 3, 7, 28, and 42 days, 100 µl samples were removed and 200 µl of methanol was added. Samples were placed on ice for 1 hr and centrifuged at 4,000 X g in an Eppendorf microfuge. The supernatant from saline and blood samples was analyzed directly by thin layer chromatography (TLC) on silica gel high performance TLC SG-60 254F plates (EM Science, Cherry Hill, NJ) by the method of HARADA *et al.* (1988, solvent system 2). Urine samples were chromatographed on C-18 Sep Pak columns (Waters, Milford, MA) equilibrated with water. The toxin fraction was eluted with 100% methanol and dried under N_2 before to TLC analysis (recovery >95% of radiolabel). Plates were scanned for radioactivity on a BID 200 radioisotope scanner (Bioscan, Washington, DC) with an efficiency of 0.6%. [^{3}H]MCYST-LR (1 mg/ml), stored in water at -10°C, was the standard. Unlabeled MCYST-LR (0.1 and 0.01 mg/ml saline) stored at 4° or -20°C was analyzed by HPLC as described above.

LD_{50} determination

Fasted mice (five animals per group) received an i.p. injection of either unlabeled or tritiated toxin. The doses were 0, 13, 20, 30, 45, 68 or 101 ug/kg toxin in saline. The LD_{50} for both forms of the toxin was calculated by the method of moving averages (WEIL, 1952). Mice were selected at random for histological analysis of liver. Either at death or 6 hr after injection and CO_2 euthanasia, tissues were removed, weighed, minced and digested in 2N KOH

for 48 hr at 65°C. Residual carcass was digested for 72 hr at 65°C. After digestion, 100 µl of each sample was added to 200 µl 2N HCl and 10 ml Hydrofluor (National Diagnostics, Manville, NJ). Radioactivity was measured in a Beckman 5800 liquid scintillation counter.

Time course of radiolabel distribution

Fasted mice were injected i.p. with 70 µg/kg [^{3}H]MCYST-LR. Two min prior to obtaining blood samples, animals were anesthetized with 50 mg/kg each Xylazine and Ketamine by i.m. injection in the hind limb. Whole blood was collected after severing the brachial artery from a skin slouch formed below the forelimb. Heparin (1000 U/ml, 50 µl) was added to the sample and plasma was collected by centrifugation at 700 X g for 10 min. Tissues were removed and treated as described above. Time points were 3, 6, 10, 15, 20, 30, 40, 60 and 90 min post-injection.

RESULTS

Characterization of [^{3}H]MCYST-LR

The chemically radiolabeled [^{3}H]MCYST-LR, as purchased, was 40% radiochemically pure. After HPLC purification, the toxin was >95% pure (Fig. 1a). The tritiated toxin exhibited the same retention time (Fig. 1b and 1c) and identical UV absorption profile (data not shown) as the unlabeled compound. The tritium label was not exchangeable after repeated treatment with acetic acid followed by evaporation under a stream of nitrogen. Complete hydrolysis of the [^{3}H]toxin yielded [^{3}H]β-methylAsp and [^{3}H]Glu 1.5:1, respectively (data not shown).

Stability of [^{3}H]MCYST-LR

Radiolabeled toxin was stable for 6 weeks in urine and saline under all temperature conditions tested (Table 1), as was the stock solution of [^{3}H]MCYST-LR (1 mg/ml, H_2O, -70°C). In whole blood, stored at room temperature, 81% of the radioactivity was associated with the MCYST-LR peak after 3 days and 54% at 1 week. When stored at 4°C for 42 days, 86% of the radioactivity co-migrated with toxin. The best storage condition was -20°C, which showed ~10% breakdown.

Toxicity of radiolabeled and unlabeled MCYST-LR

The LD_{50} of unlabled and radiolabeled MCYST-LR were 65 µg/kg (53-80, 95% confidence limits) and 76 µg/kg (65-90), respectively. The liver weights of mice that died were double those of animals that survived 6 hr post injection. Histologically, the livers from animals that received the same dose of tritiated or unlabeled toxin were indistinguishable.

The distribution of radiolabel in tissues, calculated as percent of injected dose, was similar for all doses. Figure 2 presents the data from sublethal (45 µg/kg) and lethal (101 µg/kg) doses. Heart, spleen, lung and skeletal muscle contained ~1% of the dose, liver ~50%, residual carcass ~10%, intestine (small and large) ~10%, and kidney ~1%. Recovery was 70-80%. A later experiment (see below) indicated that the unaccounted radiolabel remained in the peritoneal cavity.

Time course of radiolabel distribution

The distribution of radiolabel to liver, small and large intestine, kidney, plasma and residual carcass was examined over time after a 70 µg/kg i.p. injection of [^{3}H]MCYST-LR. At 60 and 90 min, 50% of the mice exhibited a doubling in liver weight. The percent of dose found in kidney (0.92 ± 0.08), small intestine (0.4 ± 0.3) and large intestine (2.1 ± 0.5) did not change from 3-90 min. The recovery was 70-80%. In a separate experiment, when only plasma, liver and carcass (containing all other organs) were sampled, and the liver removed such that the site of injection was not disturbed (see Fig. 3 legend), the recovery was 85-95%. Carcass and liver exhibited reciprocal time-dependent changes (Fig. 3). There was a linear accumulation in the liver for 30 min at a rate of 1.6%/min (r = 0.99) and a corresponding decrease in carcass. From 3-30 min, 5.5-8% of the dose was in plasma (Fig. 4). The elimination phase of the log plasma concentration-time curve (Fig. 4 inset) yielded a straight line (r = 0.94) with an elimination half-life of 29 min.

DISCUSSION

Chemically radiolabeled [^{3}H]MCYST-LR was shown to be indistinguishable from biologically synthesized toxin with respect to UV absorption spectra, HPLC retention time, and toxicity. Racimization of the B-methyl-D-Asp and D-Glu residues could have occurred during the labeling process. Since the configuration of the amino acids in the MCYST toxins is conserved in all the variants (BOTES *et al.*, 1982) and may be key in the toxicity of these compounds, it was important to compare the toxicity of the labeled compound and the original material. The tritium, as expected, based on the work of BOTES *et al.* (1985), was found only in the B-methylAsp and Glu residues, but,

the ratio (7.5:1 in this study versus 0.62:1, respectively) of radiolabel in the residues was different.

Quantitative recovery of radioactivity after repeated acid treatment followed by evaporation proved the tritium was nonexchangeable. The radiolabeled toxin, although stable in urine and saline, exhibited a time- and temperature-dependent breakdown in blood. The plasma concentration curve, however, showed that little toxin appeared in plasma and, therefore, analysis of blood is not a reasonable approach for diagnosis of MCYST intoxication.

Tissue distribution of radiolabel, presented as percent of injected dose, was similar for lethal and nonlethal doses (analysis 6 hr post-injection) with 50-60% accumulating in liver. GUNNEGAR *et al.* (1986) however, stated that relatively undamaged livers did not retain radioactivity 24 hr after i.p. injection of [^{125}I]MCYST-YM. This difference could be due to the later analysis time or to deiodination of the [^{125}I]compound by undamaged livers.

The time course of radiolabel accumulation in liver (70 ug/kg, LD_{50}) was linear for 30 min and reached a maximum value of 60% by 60 min. The liver was efficient at concentrating toxin and plasma contained a small percent of the injected dose. In contrast to our results, BRC KS and CODD (1987) reported an almost instantaneous (1 min) accumulation of 70% of dose (i.p., 100 ug/kg) in the liver, indicating that absorption from the peritoneal cavity was rapid. The reason for this difference is not clear.

The elimination half-life of toxin in the plasma was estimated to be 29 min, which is in agreement with the 42 min elimination phase in i.v.-injected rat (FALCONER *et al.*, 1986). Interpretation of the curve, however, was complicated by slow absorption from the site of injection. A study employing i.v. instead of i.p. injection would eliminate this difficulty.

In conclusion, [^{3}H]MCYST has proven to be an excellent tool to investigate the toxicosis of MCYST toxins. The radiolabeling procedure can be used with any of the MCYST variants since they all contain β-methylAsp and Glu in iso-linkage. The [^{3}H]toxin was stable in saline and urine and was not readily exchangeable; however, its specific activity, although more than adequate for the study described, was only 0.194 Ci/mmol. Using this material, we have begun studies on the metabolism and clearance of MCYST-LR by the perfused rat liver (PACE *et al.*, in press) and i.v.-injected mouse model.

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LEGENDS FOR FIGURES

FIG. 1. HPLC ANALYSIS OF UNLABELED AND [^{3}H]MCYST-LR. Unlabeled and [^{3}H]toxin were chromatographed on a C-18 Adsorbosphere HS column. Radioactivity was measured with a Flow I Beta detector (panel A). UV absorption at 238 nm was monitored with a Waters 490 multiwavelength detector; panel B-[^{3}H]MCYST-LR, and panel C-unlabeled MCYST-LR. The time axis for panel A was adjusted to account for the time delay between the two detectors. The peak at 13.3 min in panel C was identified as demethylated MCYST-LR (MEREISH *et al.* in press).

FIG. 2. DISTRIBUTION OF RADIOLABEL IN MOUSE TISSUE AFTER SUBLETHAL (45 µg/kg) OR LETHAL (101 µg/kg) INJECTION OF [^{3}H]MCYST-LR. At death or 6 hr post injection, organs were removed, minced and digested in 2N KOH. Radioactivity was measured by liquid scintillation and presented as percent of injected dose. Values are mean ± SEM, n=5, ■ Liver, ▩ Intestine, ▨ Carcass and ▨ Kidney.

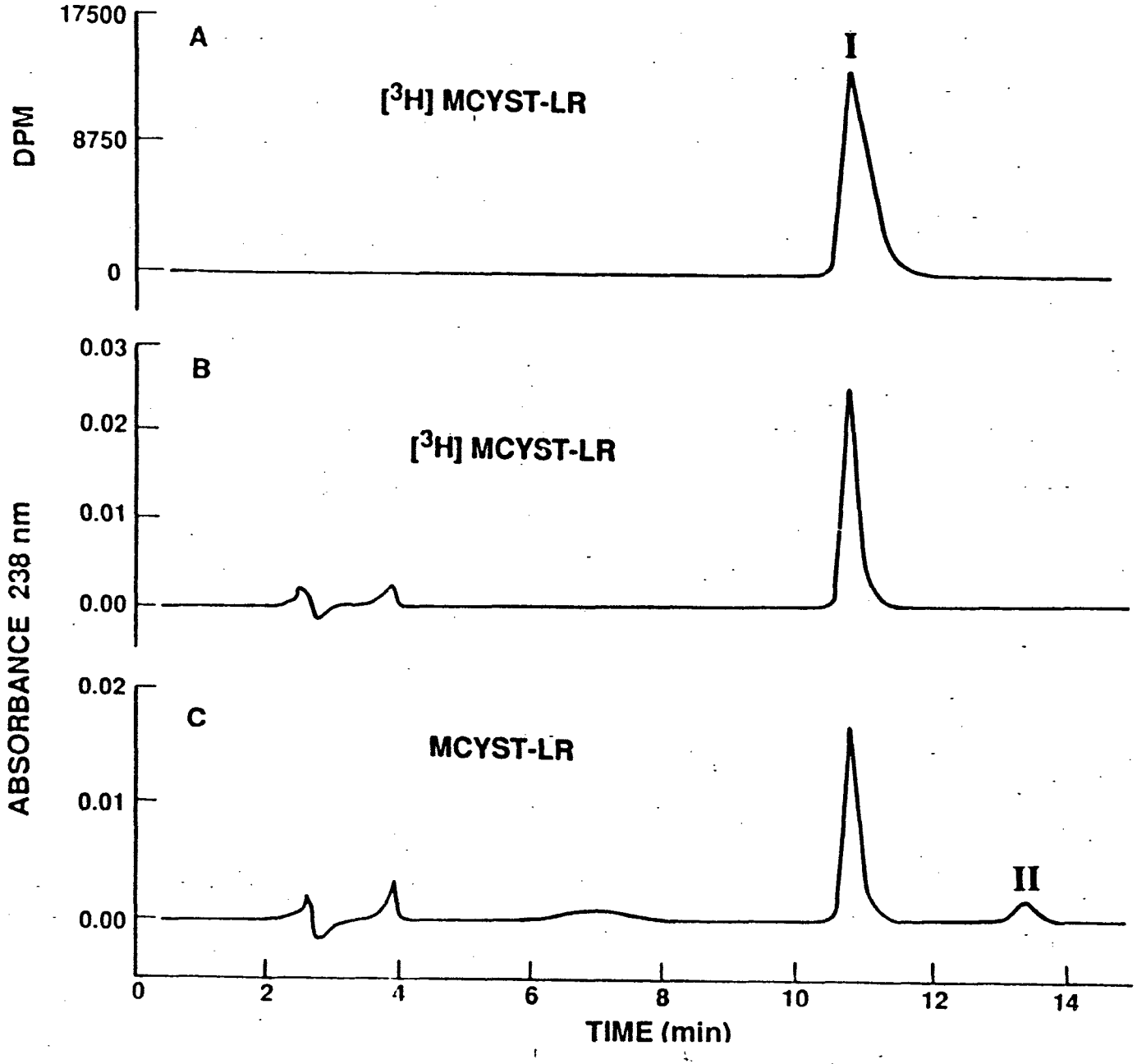
FIG. 3. TIME COURSE OF DISTRIBUTION OF RADIOLABEL IN MOUSE. Mice received an i.p. injection of 70 µg/kg [^{3}H]MCYST-LR. At 3, 6, 10, 15, 20, 30, 40, 60 and 90 min post injection, liver was removed from a horizontal incision just below the diaphragm. Liver and carcass (containing all organs except liver) were placed in 2N KOH and the radioactive content of the digest measured. Values are mean ± SEM, n=3 for carcass (△—△) and n= 9 (3 and 6 min), or 6 for liver (●—●). Data from a previous experiment in which abdominal organs were removed were included in the calculated liver values.

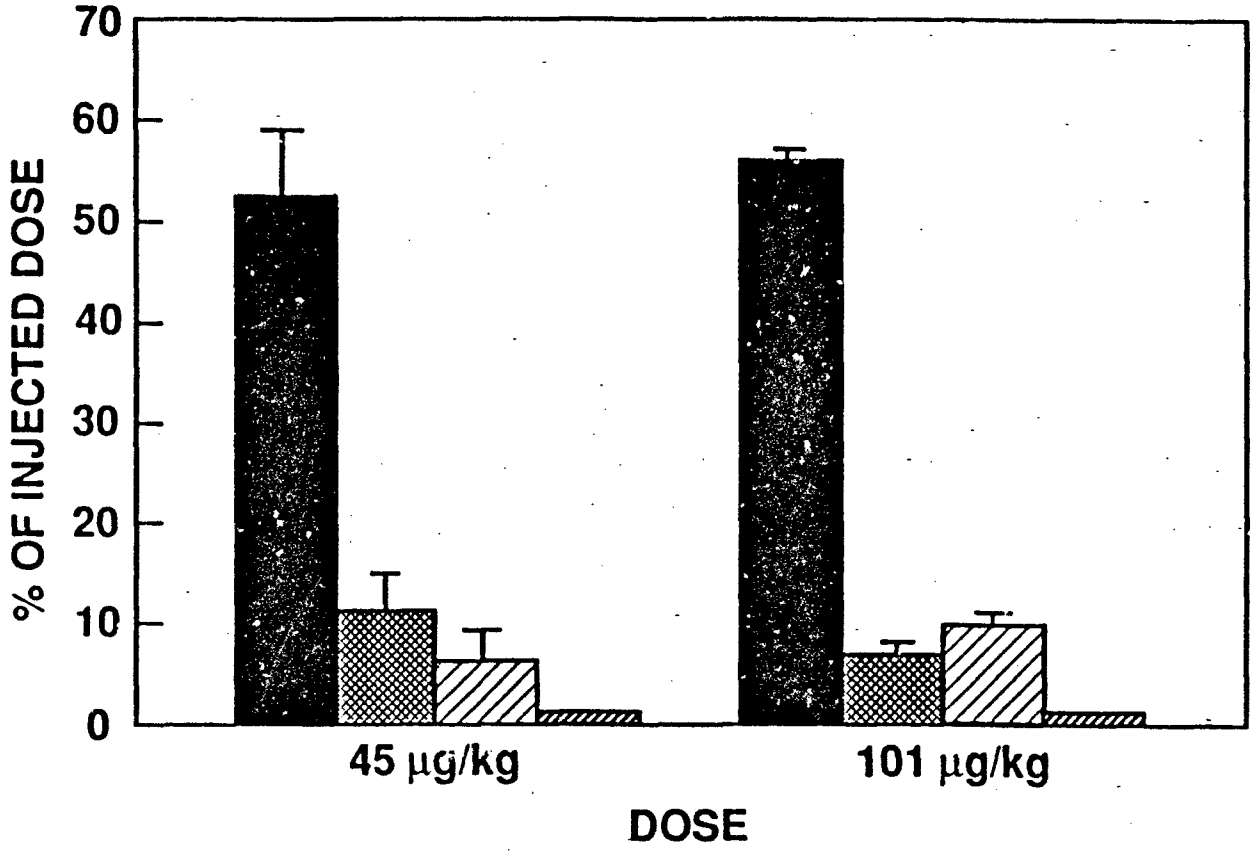
FIG. 4. TIME COURSE OF RADIOLABEL IN PLASMA. Mice were treated as described in Fig. 3. Radioactive content of plasma was measured by liquid scintillation. Values are mean ± SEM, n=9 (3 and 6 min) or 6.

TABLE 1. STABILITY OF [^{3}H]MCYST-LR IN BLOOD, URINE AND SALINE

	STORAGE TEMP (°C)	% RADIOACTIVITY THAT CO-MIGRATES WITH MCYST-LR				
		DAYS				
		1	3	7	28	42
BLOOD	22	100	81	59	37	33
	4	100	100	100	88	86
	-20	100	100	100	91	100
URINE	22	100	100	100	100	100
	4	100	100	100	100	100
	-20	100	100	100	100	100
SALINE	22	94	100	95	100	94
	4	100	100	94	100	100
	-20	100	100	100	100	100

HPLC Analysis of Tritiated and Unlabeled MCYST-LR

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10
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% OF INJECTED DOSE
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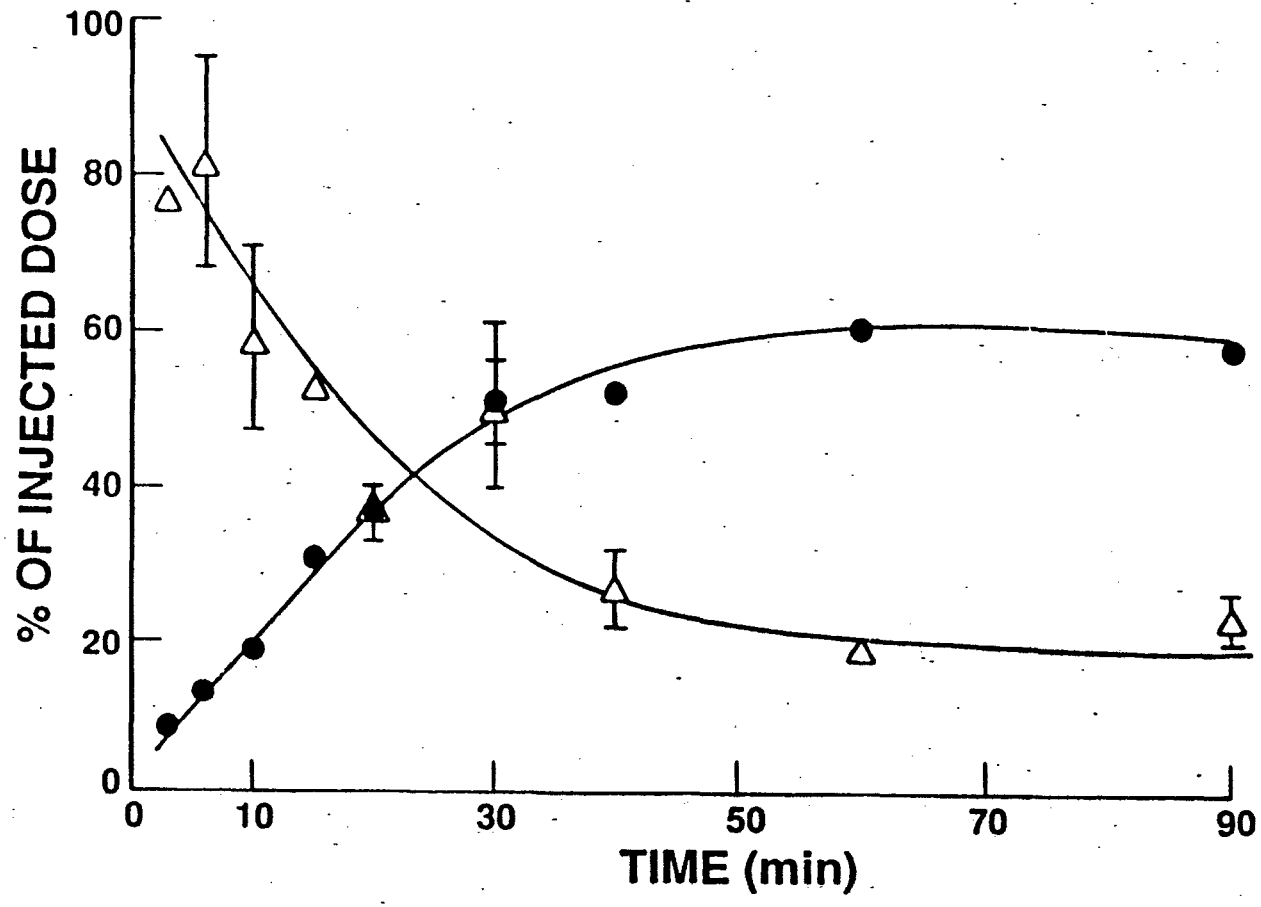
101 µg/kg
DOSE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60
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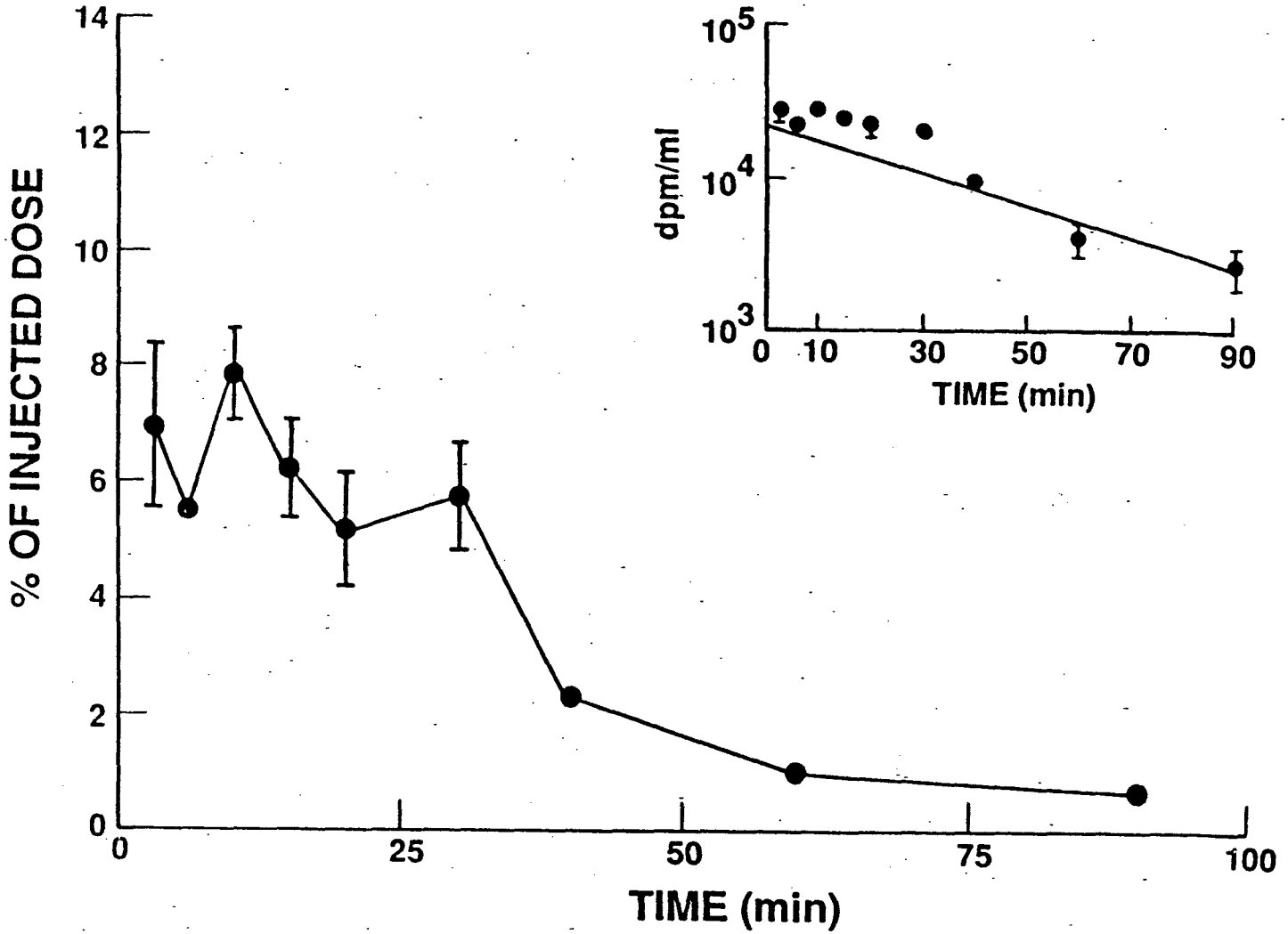
% OF INJECTED DOSE
0
10
30
50
70
90
TIME (min)


% OF INJECTED DOSE
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12
10
8
6
4
2
0
0
25
50
75
100
TIME (min)
dpm/ml
10^5
10^4
10^3
0
10
30
50
70
90
TIME (min)